## Conclusion

Protein separations can be achieved by a variety of affinity techniques, but separations in the chromatography mode are by far the most widely used. Nature defined an appropriate pathway to highly efficient separation – utilization of the phenomenon of the automatic recognition mechanism existing between a given protein and at least one other. By covalently bonding one of the pair onto an inert matrix a theoretically simple separation process can be devised. Although these immunoaffinity separations are widely practised today, severe limitations exist, not least of which are cost and instability of the affinity medium when in use. As modern design aids have become commonplace, in conjunction with newer techniques such as the development of combinatorial library arrays, it has proved possible to mimic nature and replace immunoaffinity matrices by specifically designed synthetic ligands. These new ligands not only accurately emulate the exquisite precision of the natural protein–protein interaction mechanisms, but also provide the opportunity to manipulate the ligand structures, thus offering far more efficient separations than any previously achieved. For a given protein, from whatever source and at any dilution, it is now possible virtually to guarantee that a highly cost-effective and highly efficient separation process can be developed for eventual commercial use.

Designed ligand processes have already been adopted for several very large biotechnology projects scheduled to manufacture bulk protein pharmaceuticals. A mandatory part of any new protein pharmaceutical process is the acceptance by regulatory authorities of the separation process involved. That synthesized affinity ligand separation processes have now been fully accepted by the foremost regulatory authority, the USA's Food and Drug Administration, confirms a worldwide acceptance of the power of ligand design technologies.

**See Colour Plate 1.**

## Further Reading


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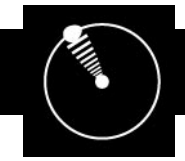
# CENTRIFUGATION



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## Introduction

Centrifugation is a mechanical process that utilizes an applied centrifugal force field to separate the components of a mixture according to density and/or particle size. The principles that govern particle behaviour during centrifugation are intuitively comprehensible. This may, in part, explain why centrifugation is seldom a part of post-secondary science curricula despite the broad range of scientific, medical and industrial applications in which this technique has been employed for well over 100 years. Applications that range from the mundane, industrial-scale dewatering of coal fines to the provision of an invaluable tool for biomedical research.

The first scientific studies conducted by Knight in 1806 reported the differences in orientation of roots and stems of seedlings when placed in a rotating wheel. However, it was not until some 60 years later that centrifuges were first used in industrial applications. The first *continuous* centrifuge, designed in 1878 by the Swedish inventor De Laval to separate cream from milk, opened the door to a broad range of industrial applications. About this same time, the first centrifuges containing small test tubes appeared. These were modest, hand-operated units that attained speeds up to 3000 rpm. The first electrically driven

centrifuges were introduced in 1910, further accelerating centrifuge development. Svedberg's invention of the analytical ultracentrifuge in 1923, operating at 10 000 rpm and equipped with transparent observation windows, marked another milestone in centrifuge technology. In the 1940s, the isolation of the first subcellular components by centrifugal techniques not only served to revolutionize our knowledge of the structure, composition and function of intracellular components, but demonstrated the potential of centrifugal methods for biomedical research. Although temporarily abandoned in 1943 in favour of a gaseous diffusion process, industrial-scale gas centrifuges were rapidly developed during World War II in an effort to enrich or separate uranium isotopes. In 1943, Pickels was the first to employ a sucrose-based density gradient to measure particle sedimentation rates. Density gradient centrifugation was further refined in the 1950s by Brakke, who applied the concept to purification and characterization of viruses, and by Anderson and co-workers at Oak Ridge National Laboratory, who designed a series of zonal centrifuge rotors for separation of subcellular particles and viruses. More recent advances have been characterized by significant improvements in materials and equipment and a broadening range of applications.

Today, centrifuges are routinely used in a variety of disciplines ranging from large-scale commercial applications to laboratory-scale scientific research. The number of centrifuge designs and configurations used in the mineral, petrochemical, chemical, medical, pharmaceutical, municipal/industrial waste, dairy, food, polymer, energy and agricultural industries (to name a few) seem almost as numerous as the applications themselves. An in-depth description of centrifuge designs and applications is, therefore, well beyond the scope of this treatise. Instead, this article will present the reader with an introduction to the theory of centrifugation, an overview of the various types of centrifugal separations, and a description of selected rotor/centrifuge designs and their more common applications.

## Theory

### Sedimentation by Gravity

A particle suspended in a liquid medium of lesser density tends to sediment downward due to the force of gravity, $F_g$. Newton showed that an object is accelerated by the gravitational force according to the relation:

$$F_g = mg = m \times 980 \text{ cm s}^{-2} \quad [1]$$

where $m$ is the mass of the object and $g$ is the acceleration due to gravity.

In an idealized case of a free-falling object being accelerated by gravity in a vacuum, the velocity of the object would exhibit a uniform rate of increase. However, for a real-world case of an object falling through air, or more appropriately for our purposes, settling in a liquid medium, there are two forces that oppose the gravitational force; the *buoyancy force*, $F_b$, and the *frictional force*, $F_f$.

**Buoyancy force** The buoyancy force was first noted by Archimedes, who showed that a particle suspended in a fluid experiences an upwards force that is equivalent to the weight of the fluid displaced:

$$F_b = m_M g = V_p \rho_M g \quad [2]$$

where $m_M$ is the mass of the fluid medium displaced, $V_p$ is the volume of the particle (=volume of the displaced fluid), and $\rho_M$ is the density of the displaced fluid.

At pressures up to several bars (1 bar = $10^5$ Pa), the buoyancy force in air or other gaseous media can be neglected to a first approximation with respect to the net gravitational acceleration experienced by solids or liquids. However, in a liquid medium, the buoyancy force is substantial. Since the volume of the settling material is equal to the volume of the fluid being displaced, the net gravitational force experienced by the particle is proportional to the difference between the mass of the particle and that of the displaced medium. Thus, assuming gravity sedimentation of a spherical particle with radius $r$ and volume of $\frac{4}{3}\pi r^3$, eqn [1] can be rewritten to show the net gravitational effect, $F_{g\text{-net}}$:

$$F_{g\text{-net}} = \tfrac{4}{3}\pi r^3(\rho_p - \rho_M)g = \tfrac{4}{3}\pi r^3(\rho_p - \rho_M) \times 980 \text{ cm s}^{-2} \quad [3]$$

where $\rho_M$ is the density of the medium (g cm$^{-3}$); $\rho_p$ is the particle density (g cm$^{-3}$); and $r$ is the particle radius (cm).

For those instances in which the medium density is greater than the density of the material in suspension, the net effect is negative, that is, particles would experience a net upward force in such instances and would tend to rise through the medium.

**Frictional force, $F_f$** In addition to the buoyancy force, the movement of a particle through a fluid medium is hindered by the viscosity of the medium, $\eta$, as described for a spherical particle by Stokes' equation:

$$F_f = 6\pi\eta r(dx/dt) \quad [4]$$

where $\eta$ is the viscosity of the medium in poise, P (g cm$^{-1}$ s$^{-1}$); $r$ is the radius of the particle (cm); and ($dx/dt$) is the velocity of the moving particle (cm s$^{-1}$).

Eqn [4] shows that the frictional force is proportional to the particle velocity and its diameter. At low velocities and pressures, the frictional force is again negligible in a gas. However, at higher velocities, even in gases, this force becomes substantial, combining with the buoyancy force eventually to exactly oppose the gravitational force, resulting in no further acceleration of the particle. This condition is known as the limiting or *terminal velocity*. Mathematically, the conditions for attaining terminal velocity are met when:

$$F_g = F_b + F_f \qquad [5]$$

The above discussion would imply that with sufficient time completely pure phases can be obtained by gravity sedimentation alone. While this may be true for the sedimentation of large particles in a medium with a significantly higher or lower density than the particle, this is not the case for smaller particles, which are impacted by diffusional forces that ultimately limit the separation efficiency as well as to other nonideality effects (see below).

**Diffusion** Random Brownian motion results in the net movement of solute or suspended particles from regions of higher concentration to regions of lower concentration, a process called diffusion. Thus, diffusion works in opposition to centrifugal sedimentation, which tends to concentrate particles. The rate of diffusion of a particle is given by Fick's law:

$$dP/dt = -DA(dP/dx) \qquad [6]$$

where $D$ is the diffusion coefficient which varies for each solute and particle; $A$ is the cross-sectional area through which the particle diffuses; and $dP/dx$ is the particle concentration gradient.

The precise impact of diffusion can be difficult and cumbersome to calculate for complex systems. It is often sufficient to keep in mind that the rate of diffusion is generally more pronounced for smaller particles than for larger ones, it increases with temperature, and its effects are lessened by higher centrifugal forces.

Aside from theoretical considerations, in a more practical sense, the time required for the settling of small to medium size particles in a gravitational field is often prohibitive. Additional obstacles to obtaining pure phases during gravity settling can also arise from attractive forces between the particles being separated and/or the medium in which they are suspended. Often, gravitational force alone is insufficient to provide the minimum force necessary to disrupt such attractions. The use of centrifugal settling addresses the shortcomings of gravity settling by shortening the time required for sample recovery at a given purity, providing a greater force for disrupting particle/particle or particle/media interactions and, within limits, lessening the detrimental effects of diffusion.

### Sedimentation in a Centrifugal Field

A particle moving in a circular path continuously experiences a *centrifugal force*, $F_c$. This force acts in the plane described by the circular path and is directed away from the axis of rotation. The centrifugal force may be expressed as:

$$F_c = ma = m\omega^2 x \qquad [7]$$

where $m$ is the particle mass (g); $a$ is the acceleration (cm s$^{-2}$); $\omega$ is the angular velocity (radians s$^{-1}$ = 2$\pi$ rpm/60); and $x$ is the radial distance from the axis of rotation to the particle (cm).

Thus, centrifugal force is proportional to the square of the angular velocity and to the radial distance from the axis of rotation. The force generated during centrifugation can be compared to the gravitational force by the *relative centrifugal force*, RCF, often referred to as the ***g*** force:

$$\text{RCF} = F_c/F_g = (m\omega^2 x)/(mg) = (\omega^2 x)/g \qquad [8]$$

Converting $\omega$ to rpm and substituting values for the acceleration due to gravity, eqn [8] can be rewritten in a more convenient form as:

$$\text{RCF} = 1.119 \times 10^{-5}(\text{rpm})^2 x \qquad [9]$$

While RCF is a ratio, and therefore unitless, it is frequently expressed in units of ***g*** to indicate the number of times that the force of the applied centrifugal field is greater than the force of gravity.

The forces acting on a particle suspended in a liquid medium in a centrifugal field are illustrated in **Figure 1**. Within the centrifugal plane, the centrifugal force acts to move particles away from the axis of rotation, while the buoyancy and frictional forces oppose this movement. The effect of the Earth's gravity can generally be regarded as negligible. Analogous to the conditions for attaining terminal velocity in a gravitational field (eqn [5]), the particle will reach a limiting or terminal velocity in a centrifugal field when the sum of the frictional and buoyancy

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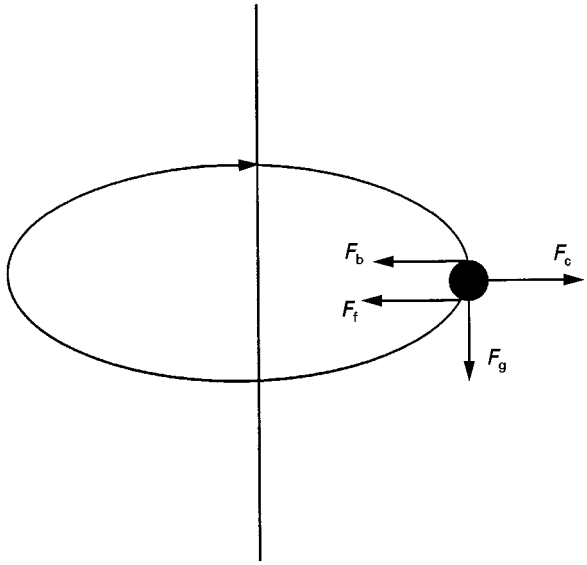


**Figure 1** Forces acting on a particle in a centrifugal field: $F_b$, buoyancy; $F_f$, frictional; $F_c$, centrifugal; and $F_g$, gravitational.

forces equals the centrifugal force:

$$F_c = F_b + F_f \qquad [10]$$

Substituting eqns [2], [4] and [7] into eqn [10] gives:

$$m\omega^2 x = V_P\rho_M\omega^2 x + 6\pi\eta r(dx/dt) \qquad [11]$$

Assuming a spherical particle and substituting $\frac{4}{3}\pi r^3$ for volume gives:

$$(\tfrac{4}{3}\pi r^3)\rho_P\omega^2 x = (\tfrac{4}{3}\pi r^3)\rho_M\omega^2 x + 6\pi\eta r(dx/dt) \qquad [12]$$

Then solving for $dx/dt$:

$$dx/dt = [2r^2(\rho_P - \rho_M)\omega^2 x]/9\eta \qquad [13]$$

Eqn [13] is more commonly expressed in terms of particle velocity, $v$, and particle diameter, $d$:

$$v = (d^2(\rho_P - \rho_M)\omega^2 x)/18\eta) \qquad [14]$$

Eqn [14] may be integrated to determine the time required for a particle to traverse a radial distance from $x_0$ to $x_1$:

$$t = [18\eta/(d^2(\rho_P - \rho_M)\omega^2)] \ln (x_1/x_0) \qquad [15]$$

where $x_0$ is the initial position of the particle and $x_1$ is the final position of the particle.

While modifications can be made to eqns [13]–[15] to account for specific rotor design, liquid–liquid, density-gradient separations, etc., these equations describe the relative impact of the more significant parameters that govern settling velocity. They show that the sedimentation rate (i.e. limiting velocity) of a particle in a centrifugal field:

- increases as the square of the particle diameter and rotor speed, i.e. doubling the speed or particle diameter will lessen the run time by a factor of four;
- increases proportionally with distance from the axis or rotation; and
- is inversely related to the viscosity of the carrier medium.

These are the fundamental premises that a practitioner must know in order to develop a rational approach to centrifugal separation.

### Sedimentation Coefficient

Since the terms $r$, $\rho_P$, $\rho_M$ and $\eta$ as given in eqns [13]–[15] are constant for a given particle in a homogeneous medium, the sedimentation rate, $dx/dt$, is proportional to $\omega^2 x$. This proportionality is often expressed in terms of the sedimentation coefficient, $S$, which is simply a measure of the sedimentation velocity per unit of centrifugal force. For a given set of run conditions, the sedimentation coefficient, $S_r$, may be calculated as:

$$S_r = (dx/dt)/(\omega^2 x) = 2r^2(\rho_P - \rho_M)/9\eta \qquad [16]$$

The sedimentation coefficient, $S$, has the dimensions of seconds and is expressed in Svedberg units equal to $10^{-13}$ s. Its value is dependent on the particle being separated, the centrifugal force and the properties of the sedimentation medium. While adequate for a given set of run conditions, it is sometimes useful to compare sedimentation coefficients obtained under differing conditions and/or sedimentation media by reference to the behaviour of the particle in water at 20°C, $S_{20,w}$:

$$S_{20,w} = s_{T,M}\eta_{T,M}(\rho_P - \rho_{20,w})/\eta_{20,w}(\rho_P - \rho_{T,M}) \qquad [17]$$

where the subscripts T and M denote the experimental temperature and medium, respectively.

### Rotor Efficiency

The time required for a particle to traverse a rotor is known as the pelleting efficiency or *k-factor*. The *k*- or clearing factor, which is calculated at the maximum rated rotor speed, is a function of rotor design and is a constant for a given rotor. *k*-Factors provide a convenient means of determining the minimum residence time required to pellet a particle in a given

rotor and are useful for comparing sedimentation times for different rotors. The $k$-factor is derived from the equation:

$$k = \ln(r_{max} - r_{min}) \times 10^{13}/(3600\omega^2)$$
$$= 2.53 \times 10^{11} \times \ln(r_{max} - r_{min})/\text{rpm}^2 \quad [18]$$

where $r_{max}$ and $r_{min}$ are the maximum and minimum distances from the centrifugal axis, respectively.

Eqn [18] shows that the lower the $k$-factor, the shorter the time required for pelleting. If the sedimentation coefficient of a particle is known, then the rotor $k$-factor can also be calculated from the relation:

$$k = TS \quad [19]$$

where $T$ is the time in hours required for pelleting and $S$ is the sedimentation coefficient in Svedberg units.

When $k$ is known (normally provided by the manufacturer), then eqn [19] may be rearranged to calculate the minimum run time required for particle pelleting.

For runs conducted at less than the maximum rated rotor speed, the $k$-factor may be adjusted according to:

$$k_{adj} = k(\text{rpm}_{max}/\text{rpm}_{act})^2 \quad [20]$$

where $\text{rpm}_{max}$ and $\text{rpm}_{act}$ are the maximum rated rotor speed and actual run speed, respectively.

$k$-Factors are also useful when switching from a rotor with a known pelleting time, $t_1$, to a second rotor of differing geometry by solving for $t_2$ in the relation:

$$t_1/t_2 = k_1/k_2 \quad [21]$$

where $t_1$, $t_2$, $k_1$ and $k_2$ are the pelleting times and $k$-factors for rotors 1 and 2, respectively.

### Deviation from Ideal Behaviour

Eqns [13] and [14] showed the relative impact on settling velocity of the more important and controllable experimental parameters. However, there are other effects that are more difficult to characterize and which can result in significant deviations from the settling velocities predicted by these equations. The most common of these effects occurs when the particles are nonspherical, as these equations are derived from Stokes' equation assuming spherical particles. For nonspherical particles, eqns [13] and [14] may be modified with a correction term, $\theta$. In Stokes' equation, the term $6\pi\eta r$ describes the *frictional coefficient*, $f_0$, for a spherical particle. The correction term, $\theta$, is calculated as the ratio of the frictional resistance, $f$, encountered by a particle of nonspherical geometry to that encountered by a sphere of the same volume, or:

$$\theta = f/f_0 = f/6\pi\eta r \quad [22]$$

The equation describing the terminal velocity for nonspherical particles in a centrifugal field may be rewritten as:

$$dx/dt = [d_e^2(\rho_P - \rho_M)\omega^2 x]/18\eta\theta \quad [23]$$

where $d_e$ is the diameter of a sphere whose volume equals that of the sedimenting particle ($d_e/2$ is the Stokes radius).

The net result of this modification is that nonspherical particles are predicted to sediment more slowly, which is a more accurate depiction of their real-world behaviour.

In addition to deviations from spherical-particle geometry, there are other effects that can lead to departure from predicted behaviour (nonideality) during sedimentation. For example, many biological particles interact with the medium via hydration, the extreme case being for those particles with osmotic properties, which can result in drastic changes in particle density and, in turn, sedimentation coefficients. Interparticle attractions, e.g. charge or hydrophobic effects, may increase the effective viscosity of the medium. In more severe cases such attractions can lead to poor separations where the centrifugal energy is insufficient to disrupt the attractions between particles that are targeted for separation. This latter effect is aggravated by the fact that the larger or denser particle will lead as the particle pair migrates toward the rotor wall while the smaller or lighter attached particle follows in its wake, and therefore experiences less frictional drag. Particles may also concentrate locally to increase the effective medium density, or form aggregates that yield complicated sedimentation patterns. Because of such deviations from ideal behaviour, equivalent sedimentation coefficients, $S^*$, defined as the sedimentation coefficient of an ideal spherical particle, are often reported for a given set of experimental conditions.

### Filtration

A mathematical description of liquid drainage from a packed bed by centrifugal forces is essentially the same as that used to describe more conventional gravity or differential-pressure filtration, the primary differences being that the centrifugal force

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or the pressure generated by the centrifugal force is substituted for the gravitational or differential-pressure terms. As filtration is an extensively characterized field of study, a description of which is beyond the scope of this article, it is recommended that the reader refer to the literature for an in-depth mathematical discussion of both conventional and centrifugal filtration. However, a brief summary of some of the more important parameters that govern flow velocity and pressure drop during centrifugal filtration follow. A simple basket centrifuge is shown schematically in **Figure 2**. Assuming a constant height of liquid within the basket, the velocity of the filtrate, $u$, through a given cake thickness, $dl$, is given by the relation:

$$u = [1/(2\pi r' H)]\mathrm{d}V/\mathrm{d}t = [1/a\mu](-\mathrm{d}P'/\mathrm{d}l) \quad [24]$$

where $H$ is the basket height or length ($2\pi r' H$ is the cross-sectional area of the filter); $r'$ is the distance from the axis of rotation to the inner cake surface; $\mathrm{d}V/\mathrm{d}t$ is the volume of filtrate passing in time $\mathrm{d}t$; $a$ is the specific resistance of the cake; $\mu$ is the viscosity of the filtrate; $\mathrm{d}P'$ is the pressure drop across a given thickness of filter cake; and $\mathrm{d}l$ is a given cake thickness.

The velocity of the filtrate through the cake and underlying filter is thus proportional to the volume of filtrate flow or the pressure differential across the filter cake, and inversely related to the surface area of the filter, filtrate viscosity and cake resistance. Eqn [24] may be rearranged and integrated to determine the total pressure drop across the cake at time $t$:

$$-\Delta P' = (a\mu/2\pi H)\,\mathrm{dV}/\mathrm{d}t \ln (r/r') \quad [25]$$

where $r$ is the distance from the axis of rotation to the outer cake surface.

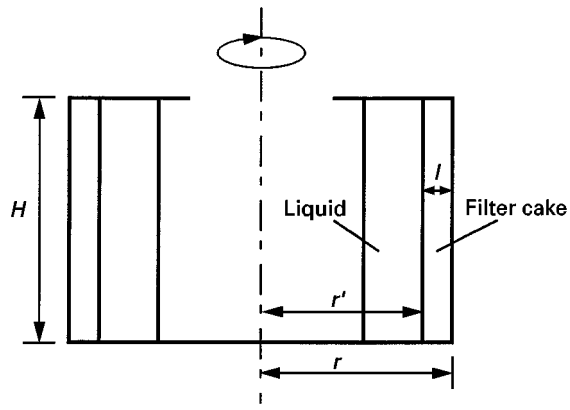


**Figure 2** Basket filtration centrifuge.

If the resistance of the filter is negligible, $\Delta P'$ is equivalent to the centrifugal pressure. A parameter that is widely used to characterize the performance of filtration equipment is the drainage number:

$$\text{Drainage number} = \bar{d}(G)^{1/2}/\mu \quad [26]$$

where $\bar{d}$ is the mean particle diameter (μm); $G$ is the centrifugal force ($=\omega^2 r/g$), where $r$ is the largest radius for a variable radius screen; and $\mu$ is the filtrate viscosity ($\mathrm{m^2\,s^{-1}}$). Higher drainage numbers correspond to more rapid drainage.

## Types of Separation

One approach to classify centrifugal separations is according to the phase of the medium and the phase of the material to be purified, e.g. gas–gas, liquid–liquid or liquid–solid. Centrifugal separations of gas-phase materials are conducted in continuous mode only, while liquid–liquid and liquid–solid may be conducted in batch, semi-batch, or continuous modes. Gas-phase separations are very important in certain applications, particularly uranium isotope enrichment, but are highly specialized and not widely used. For space considerations, gas-phase separations are omitted from this discussion. Likewise, while liquid–liquid and even liquid–liquid–solid separations are common, discussion of the separation of immiscible liquids is, for the most part, limited to the discussion of centrifuge types in subsequent sections. Suffice it to say that the principles and approaches discussed in relation to liquid–solid separations generally apply to liquid–liquid separations. That is, small droplets of a liquid dispersed in a second, immiscible liquid will behave like solid particles settling through a liquid medium until the droplets sediment and coalesce, after which the methods to remove the separated liquids from the centrifuge usually differ from those used for solids removal.

Centrifugal separations may also be classified according to the method by which purified fractions are recovered. Three modes are used: (1) batch mode, in which the total sample to be separated is processed and then recovered at the conclusion of the run by decanting the supernatant and scraping the pellet from the rotor wall; (2) semi-batch mode, in which the sample mix is continuously fed to a spinning rotor as the supernatant is continuously discharged and the pellet is permitted to accumulate for post-run removal; and (3) continuous mode, in which the sample mixture is fed continuously, the supernatant is continuously discharged, and denser liquid or solid materials are either intermittently or continuously discharged while the run is in progress.

The types of separation to be discussed focus on the separation of solids from liquid media using any of the recovery modes described above. Discussion of simpler batch-mode operation is emphasized for simplicity. Three primary types of centrifugal separations are discussed: differential sedimentation, density gradient and filtration, with density gradient being further divided into rate-zonal and isopycnic (in isopycnic separations, particles sediment until they attain a position in the gradient at which the medium density is equal to their own).

### Differential Sedimentation

As previously shown by the equations describing sedimentation (eqns [13] and [14]), larger and/or denser particles will sediment more rapidly in a centrifugal force field and will thus pellet onto the outer wall of the rotor faster than smaller or lighter particles. Most applications are based on this difference in behaviour, referred to as differential sedimentation or *pelleting*. In a simple batch-mode pelleting separation, a sample mixture called the homogenate (immiscible liquids or solid suspensions) is placed into a centrifuge container or rotor, and separated into two fractions as depicted in **Figure 3**. The unsedimented material is termed the *supernatant* and the sedimented material is the *pellet*. This approach works well when the objective is to pellet all the solid particles or to clarify the liquid. Such separations are also commonly used in the laboratory for 'quick and dirty' separations or where the objective is to enrich or clarify materials for subsequent analysis.

Obtaining high purity separations by differential sedimentation is more difficult. With respect to separating particles of similar density according to size (classification), an approximate order of magnitude difference in mass between the particles is needed for differential sedimentation to be effective. The main disadvantage of separating a homogenate in batch mode is that the centrifugal field required to pellet the larger or denser particles that are initially nearer the axis of rotation is capable of pelleting smaller or lighter particles initially closer to the outer wall (Figure 3). Product purity or recovery may be improved by either recentrifuging the supernatant to obtain more pellet, or by resuspending the pellet and recentrifuging to obtain higher purity. When purity is the primary concern, this approach can still be used as a preparatory step to provide an enriched fraction for subsequent purification. However, a more efficient one-step approach is to layer the sample mixture on top of the preloaded medium. Stopping the run before the lighter or smaller contaminant particles reach the rotor wall allows them to be decanted with the supernatant. An alternative is to use a continuous-feed rotor in which the sample mixture is introduced near the axis of rotation and the supernatant, containing the smaller or lighter unsedimented particles, is continuously discharged. A more efficient approach is to layer or feed the sample to the top of a preloaded density gradient (see below).

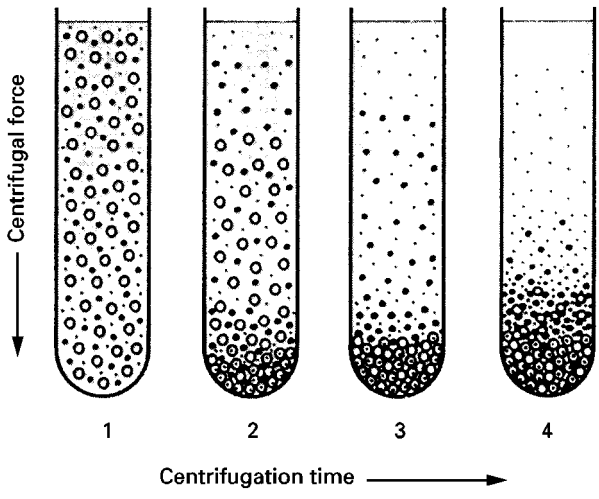


**Figure 3** Differential sedimentation or pelleting. (Courtesy of Beckman Instruments, Inc.)

### Density Gradient Centrifugation (DGC)

DGC, developed in the 1950s, also relies on differential sedimentation behaviour to separate sample components, but compensates for some of the disadvantages of homogeneous media and also allows for the simultaneous separation of multicomponent mixtures. This is accomplished by the use of a density gradient, i.e. a liquid medium that increases in density from the layers nearest the axis of rotation to those farthest away. As will be discussed, this is achieved through variation in the concentration of an aqueous solute, or other gradient material, across the rotor. With minimal precautions, density gradients are surprisingly stable for extended periods, even with the rotor stopped. DGC separations are more extensively used for smaller-scale research applications in contrast to large-scale pelleting separations that are more common to industrial applications. DGC may be conducted as either rate or isopycnic separations.

**Rate-zonal separations** This technique, also called classification, is used to separate particles of similar density according to size. For batch separations, the sample mixture is layered on top of a preloaded medium, as shown in **Figure 4**. During a rate-zonal

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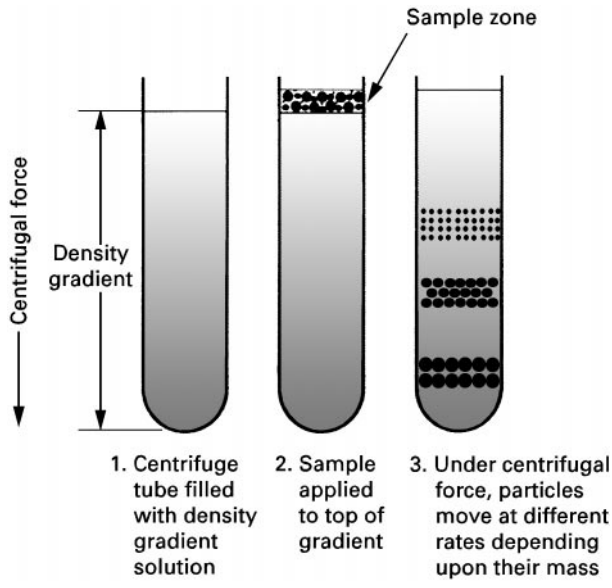
Sample zone
Centrifugal force
Density gradient
1. Centrifuge tube filled with density gradient solution
2. Sample applied to top of gradient
3. Under centrifugal force, particles move at different rates depending upon their mass

**Figure 4** Rate-zonal separation in a swinging-bucket rotor. (Courtesy of Beckman Instruments, Inc.)

separation, larger particles sediment more rapidly, just as in a pelleting run. Also similar to a pelleting run, the maximum medium density is lower than the density of the particles being processed. However, unlike pelleting runs, the run must be stopped before particles reach the bottom of the tube or rotor wall, otherwise all sample components will simply sediment to the pellet.

Rate or *setting velocity* separations may be conducted with a homogeneous medium in batch or semi-batch mode. However, the use of density-gradient media offers several advantages. The steep gradient beneath the layer of sample suppresses premature sedimentation as well as convection currents in the liquid column, both of which lower the separation efficiency. In addition, the continuous increase in density, often accompanied by an increase in viscosity across the rotor, serves to slow the faster-moving particles and provide better resolution in the sample component *bands*. Increasing-viscosity gradients also lessen diffusional effects, though this advantage may be offset by an increase in the required run time. Rate-zonal separations are well suited for mixtures of particles of similar density that exhibit two or more well-defined modes of size distribution. However, owing to the additional steps and equipment required for DGC as opposed to pelleting, DGC separations are more commonly used to separate particle mixtures based on a parameter other than size, e.g. density.

**Isopycnic separations** These separations, which are based on differences in particle densities, are conducted in a density gradient. The density range of the gradient often spans the full range of particle densities so that particles never reach the rotor wall, regardless of run time. Instead, particles move through the gradient until they reach a position in which the medium density is the same as their own (**Figure 5**). As governed by the settling velocity equations (eqns [13] and [14]), particles introduced to the top of a performed gradient sediment relatively quickly at first, with movement slowing as the difference in density between particles and gradient lessens and essentially stopping once the particles reach a position in the rotor where the density of the medium is equal to their own. Particles remain in this terminal position even after the rotor is stopped; this allows them to be recovered as density fractions. Differences in particle size only affect their rate of movement, though this may ultimately dictate the required run time. When the range of particle densities exceeds the range of the density gradient, then a mixture of pelleting and isopycnic separations will occur as some particles fully traverse the rotor and pellet while others attain their isopycnic position and remain suspended. While most density gradients are formed by the loading of solutions of successively higher density to the rotor, it is possible to form such gradients *in situ* from a homogeneous solution at high

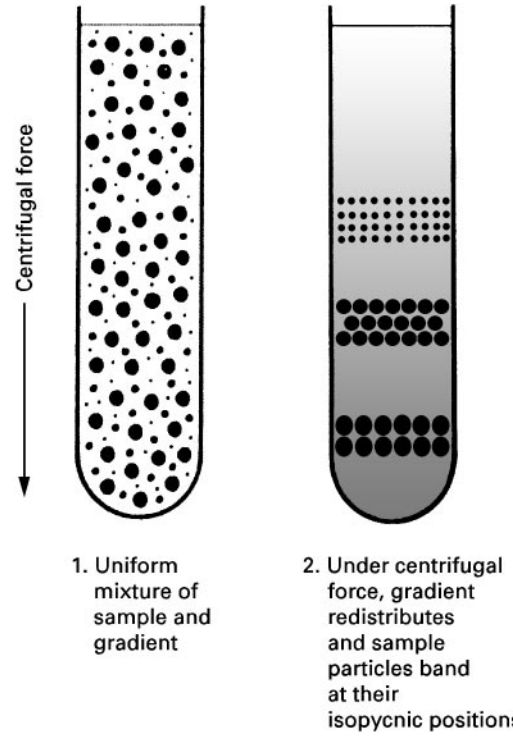
Centrifugal force
1. Uniform mixture of sample and gradient
2. Under centrifugal force, gradient redistributes and sample particles band at their isopycnic positions

**Figure 5** Isopycnic separation with a self-generating gradient. (Courtesy of Beckman Instruments, Inc.)

centrifugal speeds. This is achieved by routing the solutions to the rotor wall through veins in the central core. When such *self-generating* gradients are used, it is not necessary that the sample be layered on top of the solution but instead it may be mixed with the medium prior to loading (Figure 5). While self-generating gradients offer greater simplicity, they often require a significant increase in run time. For instance, though the advent of vertical tubes, faster centrifugal speeds, and overspeeding techniques have reduced run times to about one-third of those required only a few years ago, runs of 3 to 12 h are still typical for DNA banding experiments.

Isopycnic separation is a more powerful separation tool than rate-zonal separation in the sense that a generally greater number of particle types can be resolved. However, rate runs may still be preferred for separating large and/or fragile particles, since shorter run times and lower centrifugal forces are used. Run duration is crucial for a rate separation, whereas isopycnic runs simply require a minimum time for the particles to reach a stationary state. It is sometimes useful to conduct a two-dimensional separation in which, for instance, a rate-zonal run generates fractions of particles with similar $S$ values that are further fractionated according to density in an isopycnic separation. The reverse process can also be performed to yield particles of similar density but different particle size distributions.

**Gradient materials** The selection of an appropriate gradient material is an important consideration as the gradient properties must be compatible with the separation objectives. The desired properties of an ideal gradient material, as set forth by Griffith and by Ridge, are summarized below.

The ideal gradient material should:

- span a density range sufficient to permit separation of the particles of interest without overstressing the rotor;
- be stable in solution;
- be inert towards the fractionated materials, including biological activity;
- exert the minimum osmotic effect, ionic strength and pH;
- be removable from the product;
- be readily available and either inexpensive or easily recyclable;
- be sterilizable.

It should not:

- generate a prohibitively high viscosity;
- interfere with the assay technique (e.g. absorb UV or visible light);
- be corrosive; or
- generate flammable or toxic aerosols.

From this list of properties, it is apparent that no single ideal gradient material exists, as each separation problem imposes its own set of requirements. Rather, selection can only be made after a careful evaluation of the gradient properties with respect to the requirements imposed by the separation to be conducted. The list of materials that have been used for gradient formation is extensive with examples of the more commonly used materials along with selected properties listed in **Table 1**.

With respect to biological inertness and low viscosity, the ideal aqueous gradient material is deuterium oxide ($D_2O$). However, $D_2O$ is expensive and has a relatively low maximum density (1.11 g $cm^{-3}$).

Sucrose was used in the pioneering density-gradient work of Brakke and, due to its low cost, transparency, ready availability and nontoxic nature, is still the most widely used. Densities to 1.33 g $cm^{-3}$ can be achieved, which is sufficient for separating most cells and intracellular organelles. However, sucrose solutions are not completely physiologically inactive and often contain UV-absorbing components. Mannitol and sorbitol can be used as substitutes to

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**Table 1** Physical properties of gradient materials in aqueous solutions at 20°C (from Sheeler, 1981)

| *Gradient material* | *Tradename* | *Maximum solution concentration* | | | 20% *w/w* solution | |
|---|---|---|---|---|---|---|
| | | *Concentration* (% *w/w*) | *Density* (*g cm*$^{-3}$) | *Viscocity* (*cP*) | *Density* (*g cm*$^{-3}$) | *Viscosity* (*cP*) |
| Sucrose | | 65 | 1.33 | 182 | 1.08 | 2 |
| Sucrose polymer | Ficoll | 43 | 1.17 | 600 | 1.07 | 27 |
| Colloidal silica | Ludox-SM | – | 1.40 | – | 1.13 | 2 |
| Colloidal silica | Percoll | 23 | 1.13 | 10 | 1.11 | 8 |
| Metrizamide | | 56 | 1.44 | 58 | 1.12 | 2 |
| CsCl | | 65 | 1.91 | 1.3 | 1.17 | 0.9 |
| Polytungstate salt | LST | 85 | 2.89 | 14 | 1.20 | – |
| Polytungstate salt | SPT | 85 | 2.89 | 26 | 1.20 | 2 |

compensate for these deficiencies, but use of these sugars has disadvantages including higher viscosity and lower maximum densities. Polysaccharides also have a low osmotic pressure, but again are more viscous than sucrose solutions of equal density and may induce aggregation of the suspended sample via charge interactions.

Silica sols (e.g. Ludox™ and Percoll™), also called colloidal silica, are prepared from small silica particles in mildly alkaline solution. They provide low viscosities and osmotic pressures, even at high densities, and are transparent and inexpensive. Silica sols provide densities to 1.40 g $cm^{-3}$. Their disadvantages include a tendency to gel at pH $< 7$ and problems in complete removal from the sample. Percoll™, prepared by coating the silica particles with a polymer, eliminates the gelling problem and provides low viscosity, low osmotic pressure solutions, greater stability at low pH, and densities to 1.21 g $cm^{-3}$. However, this material is relatively expensive and removal from the sample can be a problem.

Salts are used to generate very high density aqueous solutions. Cesium chloride is by far the most widely used of this class. CsCl solutions can reach densities of $\sim 1.9$ g $cm^{-3}$ at saturation while providing a very low viscosity at lower concentrations. Although expensive, CsCl can be readily recovered and purified. CsCl solutions also have a high osmotic pressure and are corrosive, though the titanium rotors generally used with this solute are relatively resistant. CsCl gradients are commonly used in applications ranging from the separation of viruses and dense cellular macromolecules such as DNA, to geological polymers found in coal or oil shale. Other salts that have been used to produce high density gradients include sodium bromide, sodium iodide, cesium bromide, cesium sulfate, cesium formate, cesium trifluoroacetate, rubidium bromide and rubidium chloride. Though expensive, tungstate polymers such as sodium polytungstate (SPT) and lithium heteropolytungstate (LST) have recently been used to generate aqueous gradients well over 2.5 g $cm^{-3}$. Applications for these materials include the separation of graphitic carbon and mineral components from fly ash. When using such high density salt solutions, the user should be aware that at high concentration, salts may precipitate on the rotor wall, thereby generating high point densities and the potential for catastrophic rotor failure.

For nonaqueous gradients, organic liquids such as toluene, methanol or kerosene may be blended to attain gradient densities lower than that of water (1.0 g $cm^{-3}$). Of these, methanol presents an additional advantage of being water-soluble, thereby allowing gradients to be formed from a combination of the two. On the other end of the density scale, halogenated liquids such as diodomethane, bromoform and tetrabromoethane can be used to prepare very dense solutions over 2.8 g $cm^{-3}$. Problems associated with flammability, toxicity and attack of transfer lines and seals must be considered when using these materials.

**Gradient formation and shape** Gradient shape refers to the density profile across the tube or rotor as a function of gradient volume (**Figure 6**). Its choice is important as it governs the sedimentation rate in both rate and isopycnic experiments as well as the terminal position in isopycnic runs.

Gradients may be classified as *step* or *continuous*, as defined by the method of preparation. Step (discontinuous) gradients are prepared by the stepwise addition of solutions of successively higher density to the outer wall or bottom of the rotor. Steps gradients have the advantages that they may be formed without the need for a gradient generator. These gradients may also be readily *tailored* to provide larger volumes of separation media in the ranges that correspond to the density profile of the particles to be separated, thereby, permitting higher sample loadings. For continuous gradients, including the self-generating variety, the medium density varies in a continuous manner across the rotor or tube. Continuous gradients are classified as linear, exponential or isokinetic.

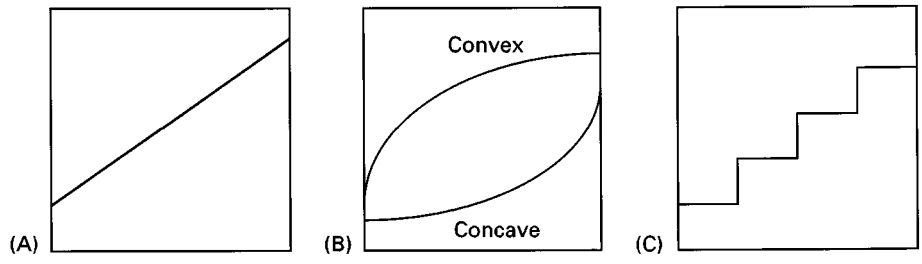


**Figure 6** Gradient shapes: (A) linear; (B) exponential; and (C) isokinetic.

In a linear gradient, density increases linearly with distance from the axis of rotation (Figure 6A), and for cylindrical swing-out rotors, with increasing gradient volume as well. In an exponential gradient, the density increases or decreases exponentially across the rotor, producing convex or concave shapes, respectively, when plotted as a function of radial distance (Figure 6B). Isokinetic gradients are designed to produce a uniform sedimentation velocity throughout the gradient by counterbalancing the increase in centrifugal force particles experience as they traverse the gradient with an increase in the density and viscosity of the medium. Such gradients are often used in analytical rotors to study sedimentation behaviour. Simple linear sucrose gradients loaded in a swinging rotor provide a near isokinetic gradient.

Various methods are used to form gradients. The simplest approach is to form the gradient *in situ*, i.e. self-generating, by mixing the sample with a single-density medium prior to loading, then forming the gradient at high centrifugal speeds. While this is the simplest approach, higher speeds and longer run times are often required. Step gradients are also easily formed by simply pumping targeted volumes of successively denser solutions to the rotor wall. Inexpensive peristaltic pumps provide the simplest means of loading step gradients. The simplest liner-gradient generators consist of two equivalent cross-section cylinders that contain an initial and a limiting solution, respectively. The chambers are interconnected at the base with liquid from the limiting solution being drawn into and mixed with the initial solution as material from the initial-solution chamber is loaded. Exponential gradient generators are similar except that the cross-sectional area of one of the chambers changes in a predetermined manner as the chambers are depleted, thereby changing the relative volume contributed from the two chambers with time. More sophisticated gradient pumps are available including mechanical pumps that use cams to mix variable amounts of low and a high density solution prior to loading or programmable pumps, e.g. a liquid chromatograph pump, to generate the targeted gradient curve shape.

Several approaches are used to analyse and/or fractionate the rotor effluent. The simplest is to split the gradient into fractions according to volume, then subsequently analyse each fraction by chemical (density, absorbance, refractive index, fluorescence) or scintillation methods. However, this approach may be somewhat limited in resolution if the collected fractions are large, and thus represent a wider range in density. An alternative approach is to route the effluent through one or more in-line, low volume flow cells to monitor the gradient properties. Automated fractionators that select cut points and automatically switch collection vessels rely on such in-line detectors.

### Analytical Centrifugation

This is the only type of centrifugal separation in which the primary objective is not to purify or dewater one or more of the feed components. Rather, this method is used to monitor particle sedimentation behaviour. Analytical centrifugation is used to characterize particle properties such as molecular weight, diffusion and sedimentation coefficients, buoyancy density, etc. The critical component in this technique is the addition of a transparent window, e.g. quartz or sapphire, to the centrifuge rotor to permit *in situ* optical measurements. Sample movement is typically monitored by UV absorption or refractive index during high speed separations in ultracentrifuges. Experiments are conducted in batch mode using very small sample volumes, as low as 5 μL for some rotors. Two classes of experiments are conducted in an analytical ultracentrifugation – sedimentation velocity and sedimentation equilibrium – anlogous to rate and isopycnic experiments in preparative ultracentrifugation. Of these, sedimentation velocity is the more common. Analytical centrifugation is less common today than in the 1950s when this was the principal method for molecular weight determinations (1–10 kDa). However, the method is still used, primarily in biological applications, for studying phenomena such as interactions between macromolecules and ligand-induced binding events. More recently, this technique has experienced somewhat of a renaissance in drug discovery applications.

### Continuous Centrifugation

These separations are similar to those previously discussed in the sense that separations are based on size or density differences. However, unlike batch-mode separation, in continuous centrifugation the sample mixture is introduced continuously to a spinning rotor as the supernatant stream continuously exits. For pelleting separations, the denser product may either accumulate on the rotor wall from where it is recovered after the rotor capacity is reached (semi-batch) or continuously discharged as the rotor spins (continuous mode). Continuous-feed centrifuges may be used for rate, pelleting, filtration, or isopycnic banding separations. They are best suited for applications in which large volumes of sample must be processed, the stream to be recovered is at low concentration, the particle sedimentation coefficient is high (less than about 50 S), or long acceleration/deceleration times are required.

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The parameters of primary concern for continuous separations are centrifugal force and flow rate. These parameters must be carefully controlled to provide sufficient time for solid or denser liquids to sediment before being carried out with the supernatant, but not so long as effectively to under utilize the throughput capacity of the rotor. The parameters controlling particle sedimentation are the same in continuous-flow as in batch-mode separations. Therefore, the maximum flow rate that can be utilized in a specific rotor at a given speed may be estimated by using eqn [15] to determine the time required for a given particle to traverse the radial distance from the rotor exit, $r_e$, and to the outer rotor wall, $r_{max}$. With information on liquid volume within the rotor and assuming laminar flow of liquid from the entry to the exit port(s), the flow rate can then be adjusted to provide this minimum residence time. The calculation of the minimum residence time is simpler if the rotor $k$-factor and the particle sedimentation coefficient are known, in which case the minimum residence time required for pelleting can be calculated from eqn [18] (i.e. $T = k/S$, where $T$ is in hours).

Continuous centrifugation is used extensively in industrial applications, where large sample throughput and recovery is more common. However, laboratory-scale continuous-feed applications are also common, particularly in semi-batch mode where the component to be isolated is present at low concentrations. Owing to the variety of continuous-flow configurations that are available, further discussion of this approach is to be found in the section on centrifugal equipment below.

### Filtration

Filtration is a mechanical means of separating solids from a liquid suspension via a porous medium or screen that permits the liquid to pass while retaining the solids. Similar to conventional filtration, achieved via a differential pressure across a filter, centrifugal filtration is driven by the pressure exerted by a liquid medium within a centrifugal force field. Opposing the centrifugal pressure is the combined resistance of the porous medium and filter cake. Centrifugal filters are commonly used to remove or recover coarse and crystalline solids from a fluid slurry, often followed by a rinse cycle to purify the solids and remove the residual mother liquor. In this technique, a sample slurry is fed to the rotor with the centrifugal pressure forcing the carrier liquid through a cylindrical screen or other permeable medium positioned around the outer wall to retain the solids or *filter cake*. The filter cake may be dried by shutting off the slurry feed and spinning the solids to attain residual moisture contents lower than generally provided by filter presses or vacuum filters. Most centrifugal filtration applications are typically conducted in continuous or semi-batch mode in which the liquids passing the filter are continuously discharged and the filter cake is continuously discharged or recovered post run. Perhaps the most widely used example of centrifugal filtration is the spin cycle in domestic washing machines.

Centrifugal filtration is a complex process that is dependent on a number of parameters including liquid viscosity, cake thickness, centrifugal force, screen area and, importantly, the size and packing characteristics of the particles themselves. Centrifugal filtration may be conducted in batch, semi-batch or continuous mode. While traditional industrial applications commonly use centrifugal filtration to recover solid materials with reduced moisture contents, many laboratory-scale spin filters, particularly in a test-tube configuration, are available. This technique is generally not amenable to broad generalizations and is, therefore, best approached on a case-by-case basis.

## Centrifugal Equipment

Centrifuges and rotors are commercially available in literally hundreds of shapes, sizes and configurations. They range from small laboratory-scale units equipped with capillary tubes, operating at speeds in excess of 100 000 rpm or forces approaching 1 000 000$\boldsymbol{g}$ to large industrial decanters that may continuously process up to 300 000 L $h^{-1}$. The primary rotor or centrifuge selection criteria must centre on the objective for conducting the separation. Parameters such as batch versus continuous; required centrifugal force and purity; throughput; the number of components to be recovered; sample toxicity/corrosiveness; time; cost; available space; noise tolerances, and so forth must be considered when selecting the appropriate centrifuge/rotor for a given application.

Early rotors were often manufactured of steel or brass, but are now more commonly constructed of aluminium and titanium. Newer carbon composites are also gaining acceptance, with plastics commonly used for small-scale applications and stainless steel for industrial-scale units. Though somewhat more expensive, titanium is particularly suitable as it has both a higher strength-to-density ratio and a high resistance to corrosion and erosion. Selected properties for steel, aluminium and titanium are shown in **Table 2**.

Centrifuge bottles and tubes are also constructed from a variety of materials. Early tubes were usually glass or stainless steel, but these have largely been replaced by plastics, e.g. polycarbonate, nylon,

**Table 2** Strength data for commonly used rotor construction materials (from Sheeler, 1981)

| *Material* | *Density* ($g\ cm^{-3}$) | *Ultimate strength* ($g\ cm^{-3}$) | *Strength: density ratio* |
|---|---|---|---|
| Aluminium | 2.79 | 2159 | 774 |
| Titanium | 4.84 | 6088 | 1258 |
| Steel | 7.99 | 7915 | 991 |

cellulose nitrate and cellulose acetate, etc. Polycarbonate is one of the more popular materials owing to its transparency and strength. The choice of material is generally dictated by the properties of the particles to be fractionated and, in high speed separations, by the maximum rated ***g*** force.

An exhaustive discussion of the many equipment options along with their advantages and disadvantages is beyond the scope of this article. Rather, a brief overview is offered of the more common centrifuge designs together with typical applications. Much of the discussion will assume batch operation, though in most cases rotors are available or may be adapted for batch, semi-batch or continuous-mode operation. However, since continuous-mode centrifuges are so widely used in industrial applications and their analogues are often unavailable in laboratory-scale units, a section describing the more common or innovative continuous-flow configurations is included.

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### Bottle Centrifuges

The most common laboratory centrifuge is the bottle centrifuge. Bottle centrifuges consist of a motor-driven vertical spindle to which a horizontal rotor, machined with an even number of sample positions (2–36), is attached. The harness and rotors are covered with a safety shield, which may also serve to reduce air friction and facilitate temperature control. Such units are normally equipped with a timer, tachometer, and manual or automatic braking. Samples may be mixed with the medium prior to loading, or layered on top of a homogeneous medium or density gradient. Bottle centrifuges are usually bench-top units that may operate at speeds up to 30 000 rpm and $g_{max}$ of 65 000, but are also available as larger, free-standing units that generate centrifugal forces in excess of 100 000***g***. Sample capacities range from capillary tube to 1 L bottles (4 L total capacity).

Bottle-centrifuge rotors classified as swinging-bucket, fixed-angle, and vertical (**Figure 7**). In the *swinging-bucket* type, the bottles are in a vertical position at rest but swing outward to a horizontal orientation as the rotor speed increases. In this orientation, the centrifugal force is applied along the length of the tube, making them suitable for rate separations. They may also be used for batch separation of immiscible liquids with some rotors specifically designed to hold separatory funnels to facilitate post-run recovery. However, their high *k*-factors make them generally unsuitable for differential pelleting, though some rotors constructed to hold short, large-diameter bottles, are designed for such purposes. *Fixed-angle* rotors are loaded and operated in a similar manner except that, as the name implies, the tube remains at a fixed angle both at rest and during the run. The fixed angle is typically 20–45° from the vertical, though *near-vertical* rotors are less than 10° from the vertical. The fixed-angle design provides a shorter pathlength (Figure 7) with a corresponding reduction in run time (lower *k*-factor). Particles that reach the outer wall of the tube during the run aggregate and quickly slide down the tube wall to form a pellet in the bottom. This makes the fixed-angle rotor useful for both pelleting (Figure 3) or isopycnic banding (Figure 4). *Vertical* rotors can be considered as an extension of fixed-angle rotors in which the angle of repose is 0° from the vertical. In this design, the maximum pathlength is equal to tube diameter, thereby providing the lowest *k*-factors for a given tube size. Vertical tube rotors are commonly used for isopycnic banding where short run times are important, as compared to near-vertical rotors, which provide short pathlengths yet permit pellet accumulation.

The tubes loaded into both vertical and fixed-angle rotors must be sealed during the run to prevent the contents from escaping as the medium moves up the outer wall at speed. O-ring sealing systems or heat sealing are commonly used. If the volume is kept sufficiently low, this step may not be necessary except to prevent the escape of hazardous aerosols, in which case a plastic screw or push-on cap may suffice.

For pelleting runs, sample recovery entails decanting the supernatant from the top and scrapping or washing the pellet into a recovery vessel or filter. For density-gradient runs, the sample may be unloaded from either the top or bottom of the tube with a pump, a Pasteur pipette, syringe, displacement liquid, etc., or by using soft plastic tubes that may be pierced to facilitate recovery of a targeted central band.

### Zonal Rotors

While bottle centrifuges can be, and are, effectively used for density-gradient centrifugation, their

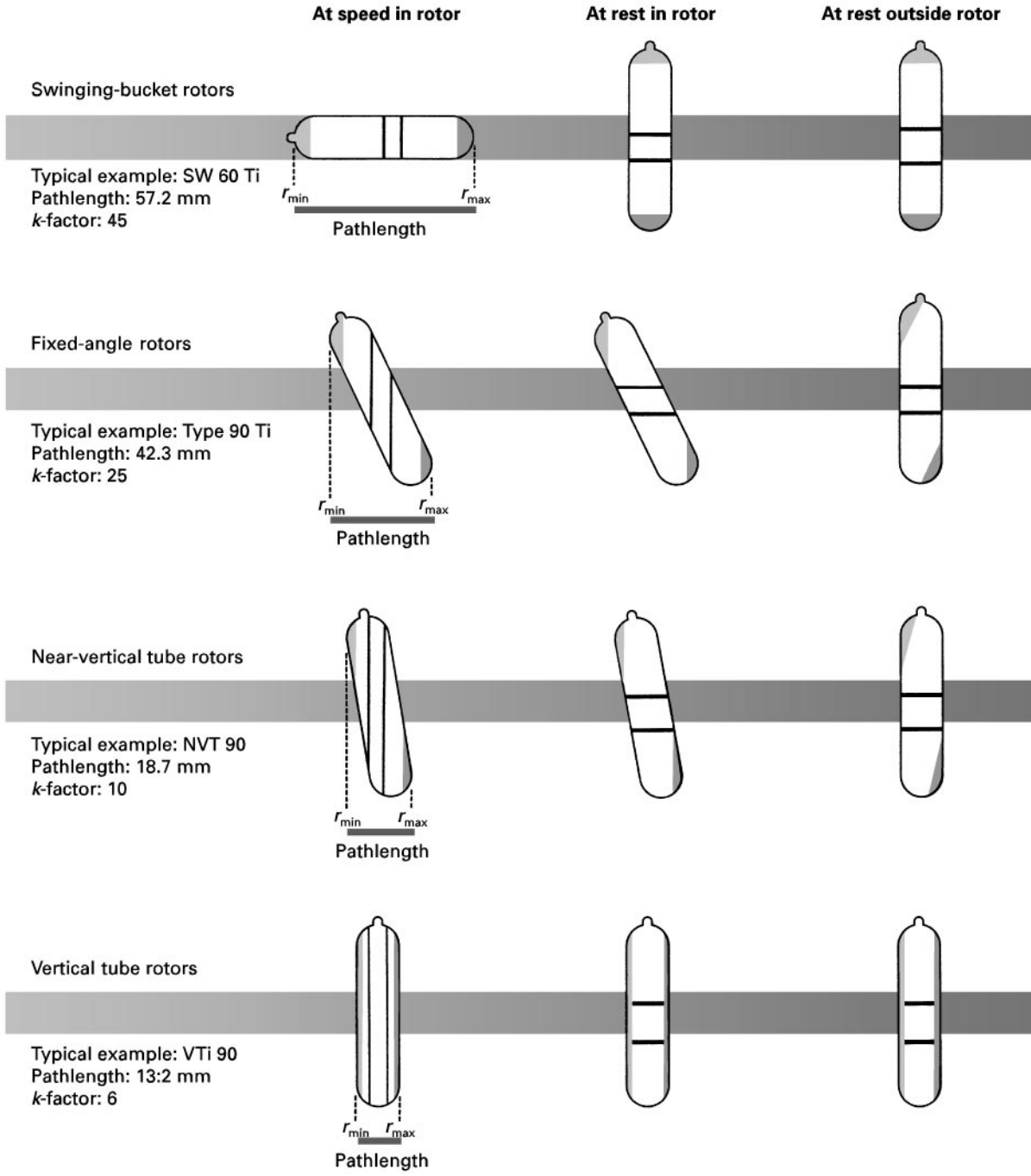


**Figure 7** Particle separation in swinging-bucket, fixed-angle and vertical-tube rotors. Dark shading represents pelleted material, light shading in floating components, and band are indicated by black lines. (Courtesy of Beckman Instruments, Inc.)

capacity may be insufficient for certain applications. This obstacle may be addressed with zonal rotors, which provide a larger internal volume for a given radius. Zonal rotors are bowls or cylindrical cavities equipped with a central core and attached vanes or septa that divide the rotor into four or more sector-shaped compartments. Zonal rotors present additional advantages over bottle centrifuges such as minimal wall effects, maximum particle and gradient resolution during sedimentation and recovery, rapid gradient formation, and high rotation speeds. Due to their higher efficiency and capacity, zonal rotors are widely utilized in applications ranging from separation/purification of proteins, viruses and subcellular components to the concentration of coal or kerogen macerals. Zonal centrifuges

can be operated in batch, semi-batch, or continuous modes and may be loaded or unloaded with the rotor stopped (*static*) or with the rotor spinning (*dynamic*).

Statically loaded and unloaded zonal rotors are also called reorienting gradient rotors. In this method, the gradient is loaded with the rotor at rest then slowly accelerated to permit the gradient to reorient from a horizontal to a vertical configuration, as illustrated in **Figure 8**. Solutions of increasing density are loaded to the bottom with the sample solution layered on top after the rotor is filled. When the rotor is accelerated, the gradient reorientates to a vertical position with the lighter fractions and sample in the centre of the rotor. After centrifugation, the rotor is slowly decelerated and the gradient returns to a horizontal orientation. The heavier fractions may be removed first by displacement with air or the rotor lid removed and the gradient pumped out. Alternatively, the gradient may be displaced with a denser liquid that forces the lighter fractions out first. The advantages of the reorienting gradient technique are simplicity and the avoidance of rotating seals that may leak or fail during dynamic loading/unloading. The major disadvantage is the tendency of the gradient to swirl as it reorients, leading to a loss in resolution.

Dynamic loading and unloading, also known as 'rotating seal', is conducted as the rotor spins, as illustrated in **Figure 9**. The gradient is pumped through a rotating seal in the centre of the rotor lid into passages machined into the rotor core, which channel the solutions to the outer wall. The lighter-density solutions are loaded first, forming a vertical layer that is displaced inward by the ensuing denser solutions. An optional high density liquid *cushion* may be added last if a reduction in the effective rotor volume is desired. The sample is introduced to the centre of the rotor by reversing the feed/exit lines. The rotor is accelerated to the operating speed for a targeted time, then decelerated to the initial loading speed. In centre unloading, a high density immiscible liquid, such as Fluorinert™, may be routed to the outer wall, forcing the gradient from the rotor, lighter fractions first. Edge unloading is similar, only a light liquid is pumped to the centre, displacing the heavier fractions first. The gradient may be fractionated as it exits by routing the effluent through a programmable fractionator that automatically switches collection vessels, or manually by selecting cutoff points with a density meter, refractometer or UV absorption cell, or by collecting predetermined volumes. While somewhat more cumbersome, dynamic loading generally provides better resolution than static loading/unloading.

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### Ultracentrifuges

'Ultracentrifuge' is an ill-defined term applied to centrifuges with rated speeds greater than about 25 000 rpm, regardless of the medium or rotor design. While speed was historically used to designate ultracentrifugation, some manufactures now reserve this term for centrifuges that operate at sufficient speeds to require a vacuum to reduce frictional drag and/or rotor heating. Most such units are also equipped with refrigerant capability for the same purpose.

Ultracentrifuges are classified as preparative or analytical. Preparative ultracentrifuges are used to separate and recover purified sample components at speeds ranging up to 150 000 rpm and forces to 900 000***g***. The rotor configuration may be any of the types described in this section – bottle, zonal, or continuous – with fixed-angle and vertical-bottle centrifuges providing the highest speeds and titanium being the most common material of construction.

Analytical ultracentrifuges, originally developed by Svedberg, are used to study the behaviour of particles during sedimentation. While analytical rotors are available in various shapes and sizes, their defining feature is a transparent window, typically constructed of quartz or sapphire, that permits the sedimenting particles to be monitored optically during the run. UV absorption and/or refractive index measurements are the most common monitoring techniques. The required sample volume is low, ranging down to 5 μL, making this a useful technique when sample availability may otherwise be a limiting factor. Sample recovery is generally a secondary consideration, if conducted at all. Analytical ultracentrifuges are available at speeds up to 70 000 rpm and centrifugal forces in excess of 350 000***g***.

### Continuous Centrifuges

Conventional batch separations are generally unsuitable for many industrial and certain laboratory-scale separations. Continuous-flow centrifugation offers certain advantages when large quantities of sample must be processed, the stream to be recovered is at low concentration, or long acceleration/deceleration times are required. Such units may be used for rate, pelleting, filtration, or isopycnic banding separations. In continuous-flow centrifugation, the sample mixture is introduced continuously to a spinning rotor as the supernatant stream continuously exits. The denser product may either accumulate on the rotor wall, from where it is recovered by stopping the run when the rotor capacity is reached (semi-batch mode), or continuously discharged during the run (continuous mode).

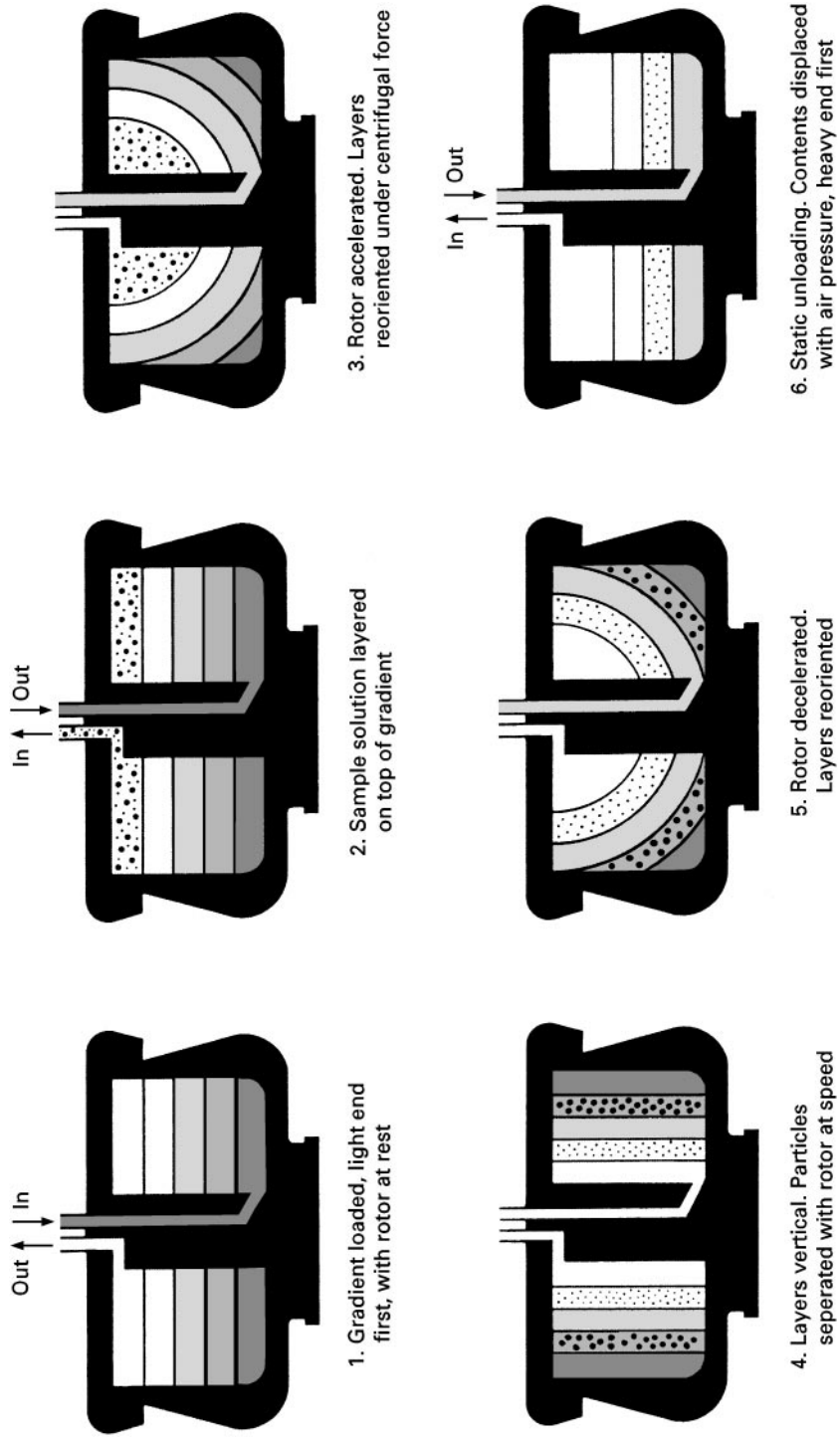
Out
In
1. Gradient loaded, light end first, with rotor at rest
In
Out
2. Sample solution layered on top of gradient
3. Rotor accelerated. Layers reoriented under centrifugal force
4. Layers vertical. Particles seperated with rotor at speed
5. Rotor decelerated. Layers reoriented
In
Out
6. Static unloading. Contents displaced with air pressure, heavy end first

**Figure 8** Static loading and unloading of a zonal rotor with a reorienting gradient core. (Courtesy of Beckman Instruments, Inc.)

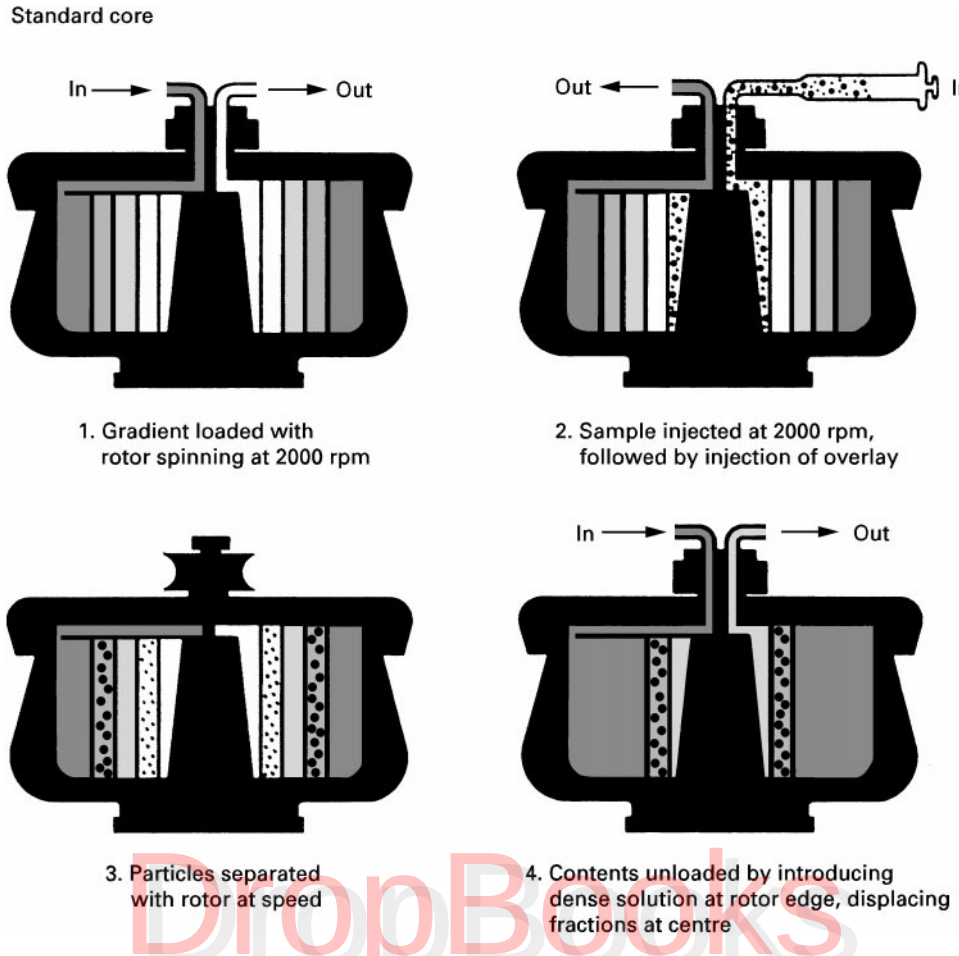
Standard core
In
Out
1. Gradient loaded with rotor spinning at 2000 rpm
Out
In
2. Sample injected at 2000 rpm, followed by injection of overlay
3. Particles separated with rotor at speed
In
Out
4. Contents unloaded by introducing dense solution at rotor edge, displacing fractions at centre

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**Figure 9** Dynamic loading and unloading of a zonal rotor. (Courtesy of Beckman Instruments, Inc.)

The rotors previously described can be, and often are, adapted for continuous-flow separations. However, the following discussion focuses on rotors that are designed specifically for continuous operation, particularly for industrial applications such as those depicted in **Figure 10**.

**Disc centrifuges** Disc centrifuges operate on the principle of differential sedimentation and are used for two-phase (liquid–solid or liquid–liquid) and three-phase (liquid–liquid–solid) separations. These are highly efficient units with some industrial-scale units generating forces of 10 000***g*** and pelleting of particles as small as 0.1 μm. Disc centrifuges are essentially a rotating bowl equipped with an internal set of conical settling plates or discs mounted at an angle to the axis of rotation (typically 30–40°). The discs serve to decrease the sedimentation pathlength and increase the sedimentation surface area, i.e. capacity factor. Denser materials sediment onto and slide across the plate surfaces before accumulating on the bowl wall (**Figure 11**) as the clarified supernatant continuously exits. In addition to the parameters of centrifugal force and flow rate, the capacity and performance of disc centrifuges are also dependent on the number, spacing and diameter of the plates. Sample mixtures may be introduced to either the interior or outside of the disc stack, depending on the nature and concentration of solids, with most units configured for liquid–liquid or liquid–liquid–solid mixtures being centre fed.

Three variations of disc centrifuges, as distinguished by their solids-handling capability, are commonly used: solids-retaining, intermittent solids-ejecting and continuous solids-ejecting (Figure 11). Solids-retaining designs (Figure 11A) are appropriate for liquid–solid or liquid–liquid separations where the solids content is less than about 1% by volume. For liquid–solid separations, the solids that accumulate on the bowl wall are recovered when the rotor capacity is reached and the centrifuge is stopped. Removable baskets are incorporated into some designs to facilitate solids removal. Recovery of two liquid streams can be achieved by positioning exit

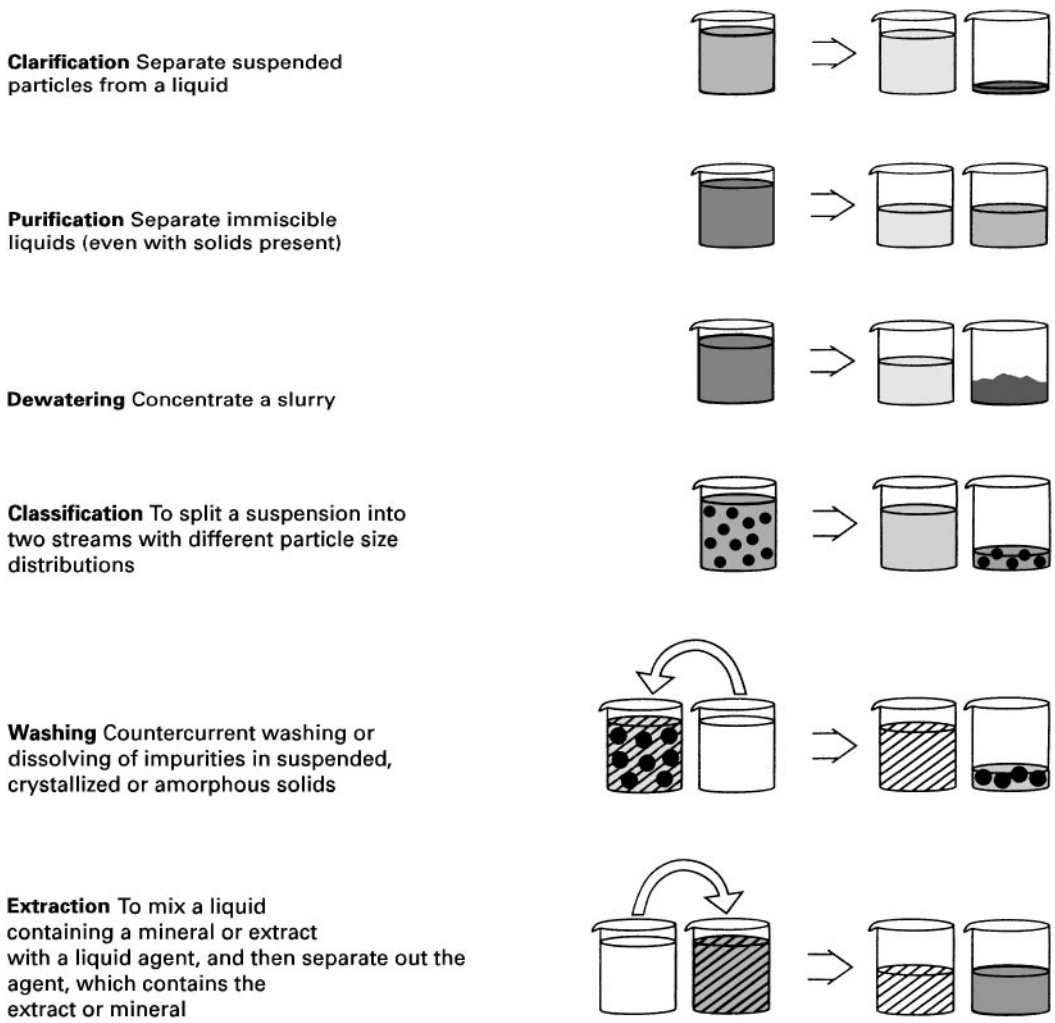
**Clarification** Separate suspended particles from a liquid
**Purification** Separate immiscible liquids (even with solids present)
**Dewatering** Concentrate a slurry
**Classification** To split a suspension into two streams with different particle size distributions
**Washing** Countercurrent washing or dissolving of impurities in suspended, crystallized or amorphous solids
**Extraction** To mix a liquid containing a mineral or extract with a liquid agent, and then separate out the agent, which contains the extract or mineral

**Figure 10** Major industrial applications for continuous centrifuges. (Courtesy of Alfa Laval Separations.)

ports at different radial distances as dictated by the relative concentration of the liquids. Commercial units are available with liquid throughput capacities of 60 $m^3 h^{-1}$ and holding capacities of 30 L. A variation on the solids-retaining disc centrifuge is the cylindrical-bowl design shown in **Figure 12**, which incorporates a series of concentric cylindrical retainers for processing liquid–solid mixtures. Unlike the disc centrifuge, in which the feed stream is split and makes a single pass through the disc stack, in the cylindrical-bowl design the liquid stream is routed through each chamber in succession, resulting in a longer residence time, more efficient recovery, and generally greater capacity (to 70 L). Applications of solids-retaining centrifuges of the stacked-disc or cylindrical-bowl design include separation of cream from milk, organic waste from water, purification of lubricating oils, or removal of water and solids from jet fuel.

Solids-ejecting stacked-disc centrifuges (Figure 11B) are more suitable for processing samples with solids contents to about 15% by volume. These units operate similarly to the solids-retaining design, only solids or sludge that accumulate on the bowl wall are intermittently discharged through a hydraulically activated, peripheral opening. Laboratory models to 18 cm diameter and industrial units to 60 cm are available, with the latter capable of throughputs in excess of 100 $m^3 h^{-1}$. Applications for these units include catalyst recovery, clarification of paints and varnishes, treatment of radioactive waste water, and copper extraction.

Continuous solids-discharge disc centrifuges, also called nozzle bowl separators (Figure 11C), are used to process samples with solids contents ranging from 5 to 30% by volume. In this design, solids are continuously discharged via backward-facing orifices, i.e. nozzles, closely spaced around the outer periphery of

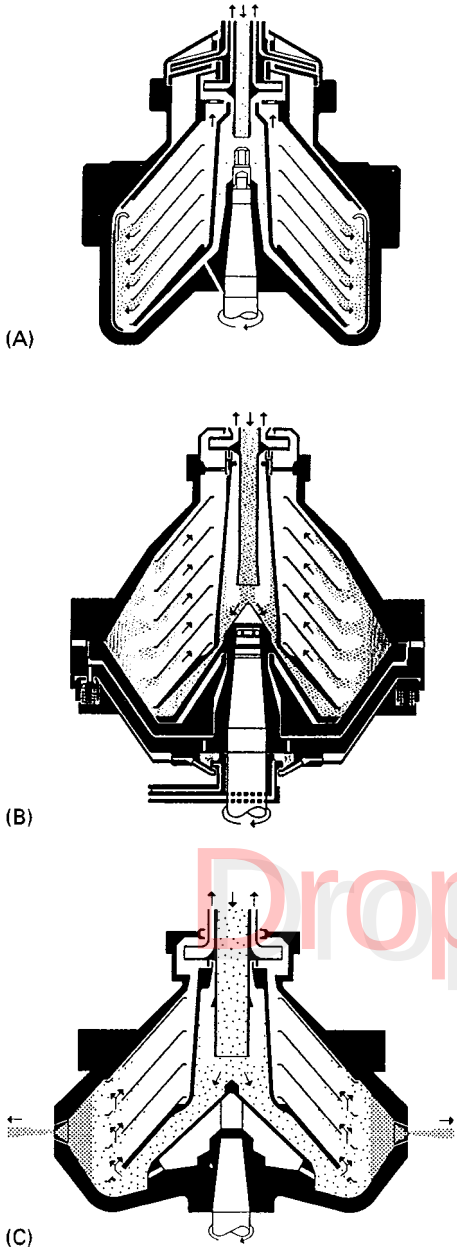


**Figure 11** Disc centrifuge configurations: (A) solids-retaining; (B) intermittent solids-ejecting; and (C) continuous solids-ejecting. (Courtesy of Alfa Laval Separations.)

the bowl. Due to the high discharge velocities resulting from the centrifugal pressures, nozzle erosion can occur. Thus, the materials used for nozzle construction and the ease of replacement of eroded components should be considered. Newer designs discharge to an internal chamber where the discharge is pumped out as a product stream. Industrial units are available to 200 $m^3 h^{-1}$ throughput capacity, elevated temperature ($\leq$200°C) or pressure (7 bar) capability, and particle removal to 0.1 μm. Applications for continuous-discharge disc centrifuges include production of baker's yeast, dewatering of kaolin clay, titanium dioxide classification, and coal-tar and tar-sand clarification.

**Continuous conveyor discharge** These centrifuge types integrate an active mechanical solids discharge mechanism in an imperforate bowl for the continuous processing of larger sample volumes. The bowl shape is tubular, having a length-to-diameter ratio of 1.5–5.2, and may operate in either a horizontal or vertical configuration. The vertical configuration is generally preferred for reduced or elevated temperature and/or pressure applications owing to fewer mechanical problems with seals and heat expansion. The solids-discharge mechanism is most commonly, a helical screw turning at a slightly slower rate than the rotor, though pistons or conveyer belts are also used. **Figure 13** illustrates a helical-screw configuration used for three-phase separations (liquid–liquid–solid). Solid–liquid and liquid–liquid configurations with either concurrent or countercurrent flow regimes are commercially available. Such mechanical discharge units typically operate at lower centrifugal forces (to 5000***g***) than disc centrifuges. However, they are capable of very high throughput, up to 300 000 L $h^{-1}$, and can be used to process feed streams containing up to 50% solids by volume. While a limited number of industrial units operate on materials smaller than 1 μm, particles smaller than about 2 μm are usually not collected in such units, a characteristic that is used to advantage for particle classification. Continuous conveyer centrifuges are widely used in the chemical, mining, pharmaceutical,

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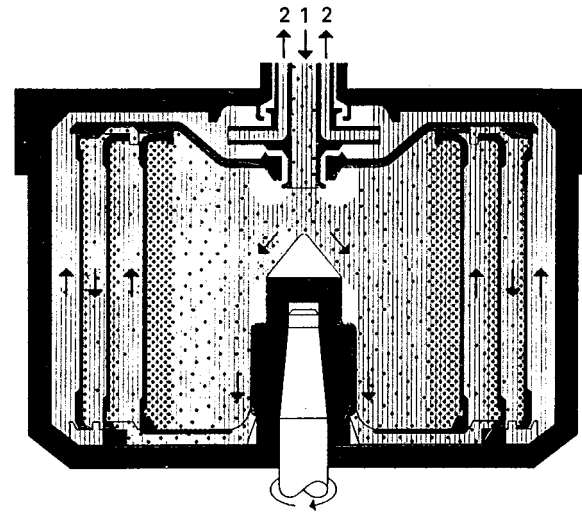


**Figure 12** Schematic of a cylindrical-bowl centrifuge. (Courtesy of Alfa Laval Separations.)

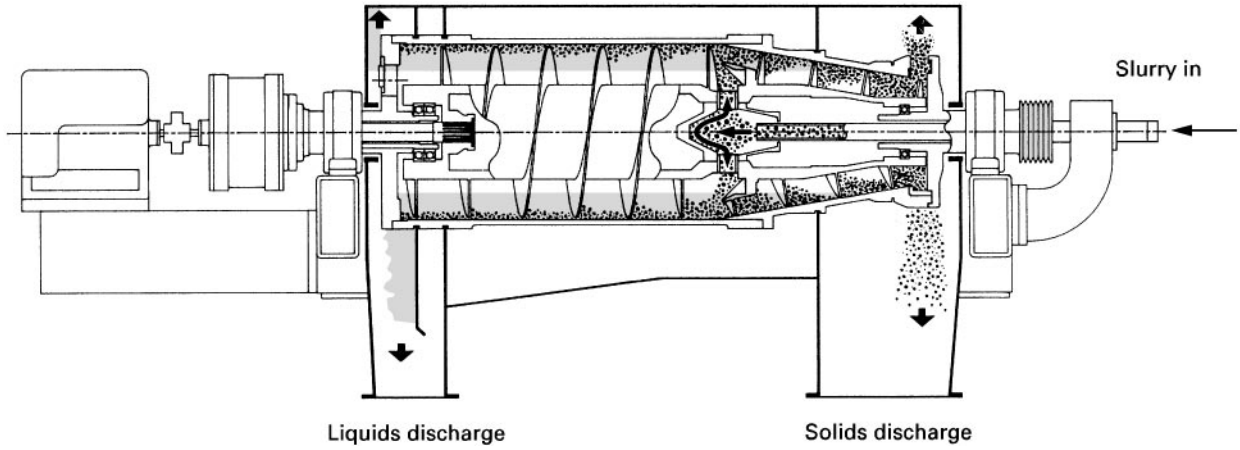


**Figure 13** Schematic of a horizontal continuous-conveyer centrifuge. (Courtesy of Alfa Laval Separations.)

biotechnology and food sectors for clarifying, classifying, dewatering and thickening applications.

**Tubular centrifuges** These centrifuges utilize a vertically mounted, imperforate cylindrical-bowl design to process feed streams with a low solids content. Liquid(s) is discharged continuously and solids are manually recovered after the rotor capacity is reached. One configuration, designed for recovery of two immiscible liquids and a solid product, is shown in **Figure 14**. Other configurations for processing solid–liquid or liquid–liquid mixtures are also widely used. Industrial models are available with diameters up to 1.8 m, holding capacities up to 12 kg, throughput rates of 250 $m^3 h^{-1}$, and forces ranging up to 20 000*g*. Laboratory models are available with

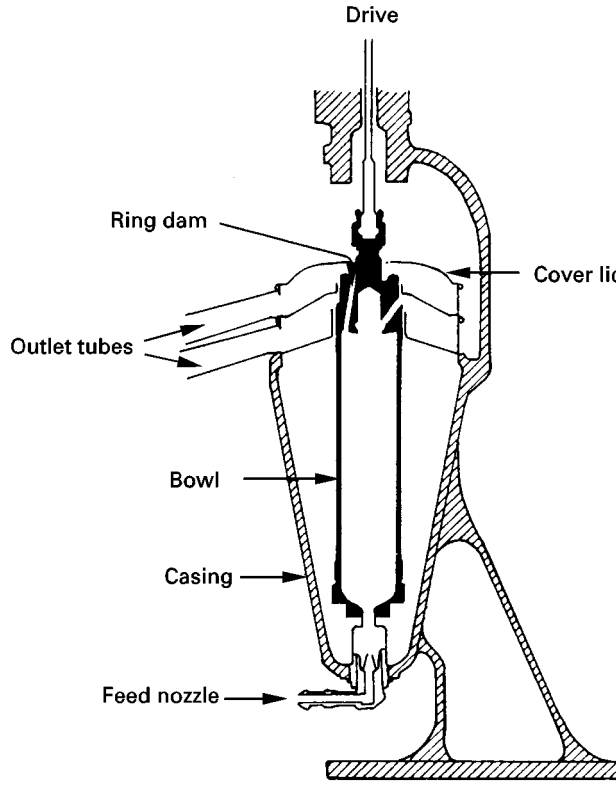


**Figure 14** A tubular centrifuge configured for recovery of two liquids and one solids stream. (Courtesy of Alfa Laval Separations.)

diameters of 4.5 cm, throughput rates of 150 L $h^{-1}$, and centrifugal forces ranging up to 62 000*g*. Because of their high speed and short settling path, tubular centrifuges are well suited for the pelleting of ultrafine particles, liquid clarification, and separation of difficult-to-separate immiscible liquids. In addition to the standard electric motor used for most laboratory centrifuges, laboratory-scale tubular centrifuges are available with turbine drives. Tubular centrifuges were refined for the separation of penicillin during World War II but since then have largely been replaced by disc centrifuges because of their limited holding capacity. However, they are still widely used for applications that involve the efficient recovery of high value products at high purity, especially in the pharmaceutical and chemical industries. Typical applications include recovery of *Escherichia coli* cells and flu viruses, removal of colloidal carbon and moisture from transformer oils, removal of small particles from lubricating oils, blood fractionation, and de-inking.

**Continuous zonal rotors** Zonal rotors are often used for smaller scale, semi-batch separations. Operation is similar to that previously described for batch separation only a larger diameter core with a different flow pattern is inserted as illustrated in **Figure 15**. Continuous-feed separations in zonal centrifuges are best suited for low concentration, high volume samples. Such separations may be conducted with a homogeneous medium for sample pelleting, or with a density gradient for materials that may be adversely affected by pelleting (e.g. viruses that may lose their activity) or if simultaneous isolation of two or more materials is desired. Applications include purification of viruses from tissue-culture media, harvesting bacteria, or separating fine clay particles in water pollution studies.

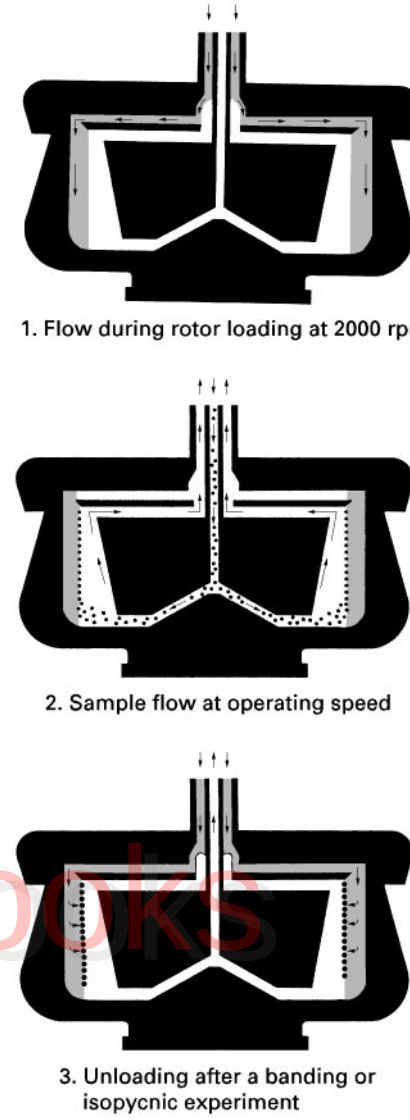
1. Flow during rotor loading at 2000 rpm
2. Sample flow at operating speed
3. Unloading after a banding or isopycnic experiment

**Figure 15** Flow regimes in a continuous-flow zonal rotor. (Courtesy of Beckman Instruments, Inc.)

**Elutriation rotors** Another type of laboratory-scale continuous-flow centrifugation is elutriation or counterstreaming, used to separate particles with differing sedimentation rates (rate separation). A schematic of the elutriation process is shown in **Figure 16**. Conical or funnel-shaped rotors are used with the small end positioned farthest from the axis of rotation. The rotor is initially filled with a buffer solution followed by the sample mixture, introduced at a constant rate to the small end of the spinning rotor, where particles experience the opposing forces of the centrifugal field and the flowing medium. Initially, the frictional force of the carrier medium is greater than the centrifugal force and all particles are swept inward by the flowing carrier. However, as the entrained particles migrate toward the large end of the chamber, the linear velocity of the carrier decreases as the cross-sectional area of the rotor increases. Due to the greater sedimentation rates for larger particles in a centrifugal force field, smaller particles continue to migrate toward the centre of the rotor while larger particles remain suspended or move more slowly, resulting in particle classification. Such separations are semi-batch since, as the concentration of larger particles in the rotor increases to capacity, sample feed must be stopped so that these particles may be eluted with a higher velocity rinse solution. Elutriation rotors typically operate at lower centrifugal forces (10 000*g*) with throughputs to 400 mL $min^{-1}$. A common application is the isolation of specific cell types.

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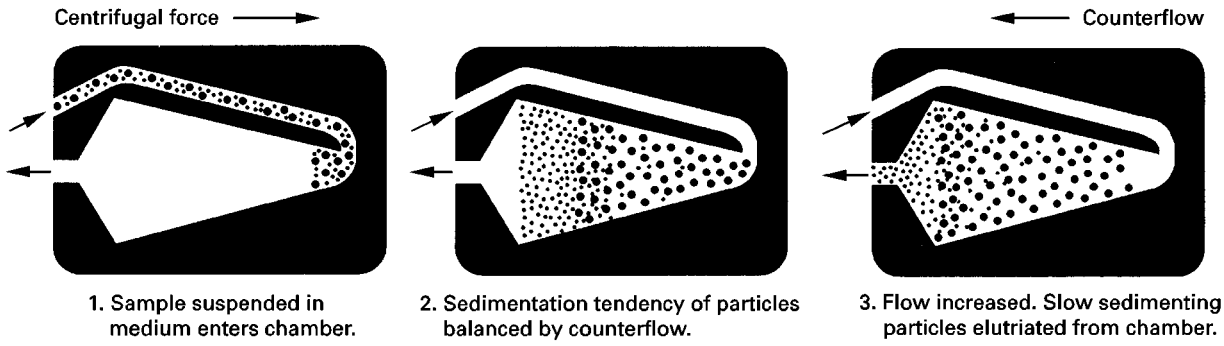
Centrifugal force
Counterflow
1. Sample suspended in medium enters chamber.
2. Sedimentation tendency of particles balanced by counterflow.
3. Flow increased. Slow sedimenting particles elutriated from chamber.

**Figure 16** The elutriation process. (Courtesy of Beckman Instruments, Inc.)

### Centrifugal Filtration Equipment

In centrifugal filtration, centrifugal force is used to press a solids suspension against a filter medium that permits the mother liquor to pass while retaining the solid particles. Such centrifuges are used for the separation of solids from liquid slurries, chiefly in industrial applications, and are usually characterized in terms of the final moisture content, drainage time and centrifugal force. In addition to the centrifugal field, the drain or screen area and cake thickness are the primary controllable parameters that govern performance. Filtration centrifuges are available in numerous configurations with units often designed or modified for a specific application. Three of the more common designs are batch/semi-batch basket centrifuges, continuous push-type and continuous conical centrifuges.

**Basket centrifuges** The simplest and most common centrifugal filtration units are basket centrifuges. They are particularly useful when the nature or concentration of the solids varies substantially with time or for the recovery of small or difficult-to-filter particles. Basket centrifuges incorporate a perforated cylindrical bowl that is lined with a filtration medium, usually a fabric or metal screen. Industrial units generally spin at relatively low rates ($<$4000 rpm), are available with bowl diameters ranging from 0.3 m to 2.4 m, and may be operated at elevated temperatures (350°C) and/or pressures (1 MPa). The slurry is fed to the centre of the basket with the mother liquor passing and the cake accumulating against the filtration medium. When the accumulated cake volume is sufficient either to retard further filtration or unbalance the centrifuge, the solids must be discharged. This is achieved in one of three ways: (1) the centrifuge is stopped and the cake is manually scraped, useful for smaller batches when production does not warrant the additional costs of automation, for processing different materials in a single unit, or when the equipment must be sterilized between batches; (2) the cake is mechanically unloaded at reduced speed by using a single or multiple plow; or (3) the cake is continuously removed at speed with a hydraulic *knife* in a *peeler* centrifuge, most useful for moderate production rates and for materials that drain freely. Other basket centrifuges, termed *inverting filter* centrifuges, have flexible filters that may be inverted to discharge the accumulated solids.

Continuous centrifugal filters are more useful for higher volume processing of fast-draining solids in applications that do not require a low level of moisture in the recovered product. They can be further divided into push-type (cylindrical) and conical filters.

**Push-type centrifugal filters** These units consist of a rotating cylindrical drum that incorporates a feed funnel that rotates with the drum. The slurry is introduced via the feed funnel where it is accelerated before being deposited to one end of the drum. Liquids pass through a cylindrical screen under centrifugal pressure as the solids accumulate to form a cake. The cake is then pushed by a reciprocating piston toward the exit located at the opposite end of the drum. Push-type filters may be single or multiple stage, with the latter incorporating a cylindrical screen with two to six variable-diameter steps. The diagram of a multistage push-type filter in **Figure 17** illustrates the integration of filtration and rinse cycles in a continuous operation.

**Conical centrifugal filters** In a conical centrifugal filter, the slurry is introduced to the small end of a conical drum, which supports the filtration medium. Liquids drain through the drum filter as the solids are either mechanically or self-discharged through the large end. The movement of the solids from the small end of the cone to the larger-diameter end results in a thinning of the cake that facilitates

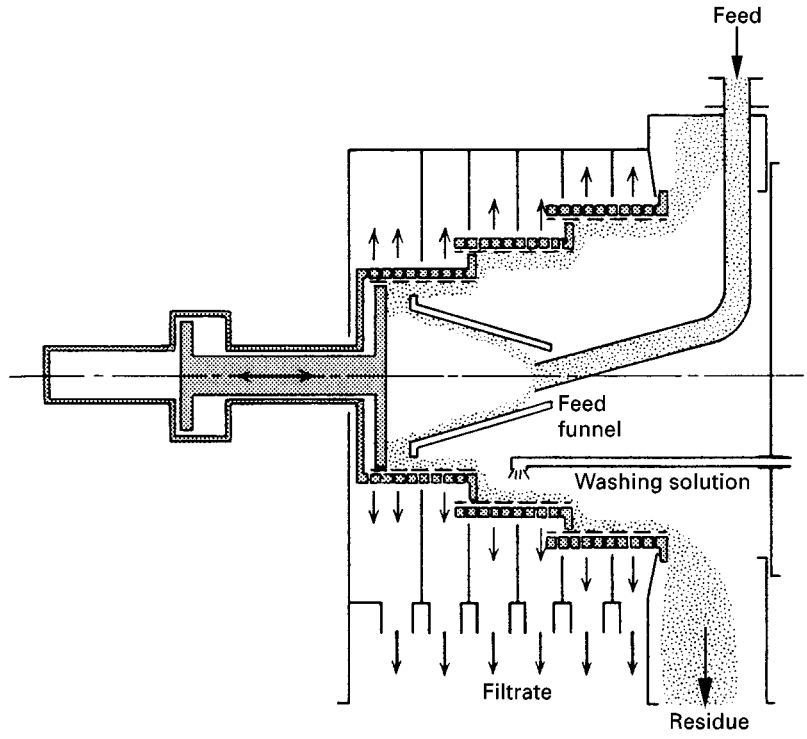


**Figure 17** Multistage push-type centrifugal filter. (Courtesy of Alfa Laval Separations.)

drainage. Some designs incorporate a lower cone angle at the small end, where most of the drainage occurs, and a higher angle on the large end, to increase the solids-holding capacity and provide additional drainage time.

Three methods of solids removal are commonly used for conical filtration: screw conveyer, self-discharging or vibratory. Screw conveyers consist of a vertical or horizontal conical bowl with an internal helical screw rotating slightly faster than the conical drum. In this configuration, solids are continually moved from the small end of the cone and discharged from the larger end. Screw-conveyer units have cone angles that generally range from 10–20°, feed capacities of 1–15 $m^3\,h^{-1}$, and centrifugal forces to 3500***g***. Applications include the dewatering of crystalline solids and the extraction of solids from fruit and vegetable pulps. Self-discharging filters are similar to screw conveyers, only the cone angle is larger (20–35°) than the angle of repose of the cake. At these greater angles, the solids slide down the tapered walls and exit the large end of the conical drum without the need for mechanical assistance. Vibratory-discharge filters are also similar in design to screw-conveyer units, but in this case solids discharge is accomplished by applying a vibratory or oscillatory motion to the bowl or casing. Such units are typically operated at low speeds (300–500 rpm) and used to process larger particles (0.25–30 mm) than screw-conveyer or self-discharging designs. The cone angle is 13 to 18° with throughput capacities of 25–150 $t\,h^{-1}$. Their most common application is for the dewatering of coal fines.

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**See Colour Plate 2.**

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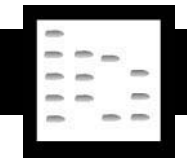
# CHROMATOGRAPHY



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## Introduction

Chromatography is the most widely used separation technique in chemical laboratories, where it is used in analysis, isolation and purification, and it is commonly used in the chemical process industry as a component of small and large-scale production. In terms of scale, at one extreme minute quantities of less than a nanogram are separated and identified during analysis, while at the other, hundreds of kilograms of material per hour are processed into refined products. It is the versatility of chromatography in its many variants that is behind its ubiquitous status in separation science, coupled with simplicity of approach and a reasonably well-developed framework in which the different chromatographic techniques operate.

Chromatography is essentially a physical method of separation in which the components of a mixture are separated by their distribution between two phases; one of these phases in the form of a porous bed, bulk liquid, layer or film is generally immobile (stationary phase), while the other is a fluid (mobile phase) that percolates through or over the stationary phase. A separation results from repeated sorption/desorption events during the movement of the sample components along the stationary phase in the general direction of mobile-phase migration. Useful separations require an adequate difference in the strength of the physical interactions for the sample components in the two phases, combined with a favourable contribution from system transport properties that control sample movement within and between phases. Several key factors are responsible, therefore, or act together, to produce an acceptable separation. Individual compounds are distinguished by their ability to participate in common intermolecular interactions in the two phases, which can generally be characterized by an equilibrium constant, and is thus a property predicted from chemical thermodynamics. Interactions are mainly physical in type or involve weak chemical bonds, for example dipole–dipole, hydrogen bond formation, charge transfer, etc., and reversible, since useful separations only result if the compound spends some time in both phases. During transport through or over the stationary phase, differential transport phenomena, such as diffusion and flow anisotropy (complex phenomena discussed later), result in dispersion of solute molecules around an average value, such that they occupy a finite distance along the stationary phase in the direction of migration. The extent of dispersion restricts the capacity of the chromatographic system to separate and, independent of favourable thermodynamic contributions to the separation, there is a finite number of dispersed zones that can be accommodated in the separation. Consequently, the optimization of a chromatographic separation depends on achieving favourable kinetic features if success is to be obtained.

## The Family of Chromatographic Techniques

A convenient classification of the chromatographic techniques can be made in terms of the phases employed for the separation (**Figure 1**), with a further subdivision possible by the distribution process employed. In addition, for practical utility transport processes in at least one phase must be reasonably fast; for example, solid–solid chromatography, which may occur over geological time spans, is impractical in the laboratory because of the slow migration of